Virology **295,** 272–283 (2002)
doi:10.1006/viro.2002.1373, available online at http://www.idealibrary.com on IDEAL®

An Altered Cellular Response to Interferon and Up-Regulation of Interleukin-8 Induced by the Hepatitis C Viral Protein NS5A Uncovered by Microarray Analysis

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Received November 13, 2001; returned to author for revision December 19, 2001; accepted January 16, 2002

There is evidence for an inhibition of interferon-α antiviral activity by the hepatitis C viral protein, NS5A. To identify the mechanisms through which NS5A blocks interferon activity, we compared the gene expression profile of interferon-treated Huh7 cells, stably expressing NS5A with control, using microarrays. Following interferon treatment, 50 genes were up-regulated by at least twofold in control clones, whereas induction of 9 of the 50 genes was significantly reduced in NS5A-expressing clones. The strongest effect of NS5A on interferon response was observed for the OAS-p69 gene. Remarkably, Huh7 cells expressing NS5A showed an up-regulation of interleukin-8. Up-regulation of interleukin-8 was also observed upon transient expression of NS5A mutants isolated from patients responsive or resistant to interferon therapy. Addition of interleukin-8 to Huh7 cells inhibited the antiviral activity of interferon and, similarly to NS5A, reduced the induction by interferon-α of selective genes including OAS-p69. Our findings provide a mechanism for NS5A-mediated interferon resistance.

Key Words: HCV; IFN-α; NS5A; IL-8; microarray.

INTRODUCTION

Hepatitis C virus (HCV) is a positive-stranded RNA virus classified in the *Flaviviridae* family, which exhibits marked viral heterogeneity (Brechot, 1996). The polyprotein precursor is co- and posttranslationally processed to yield mature, structural (C, E1, and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (Bartenschlager *et al.,* 1993; Grakoui *et al.,* 1993; Selby *et al.,* 1993). HCV is a highly prevalent pathogen (Alter, 1997) and 70–80% of acutely infected subjects will become chronic carriers, with a high subsequent risk of progression to liver cirrhosis and hepatocarcinoma (Di Bisceglie, 1995). Interferon-α (IFN-α) therapy ($\pm$ ribavirin) is the only currently available treatment for HCV, but is not effective for a majority of patients (McHutchinson *et al.,* 1998; McHutchinson and Poynard, 1999; Schalm *et al.,* 1999). Several studies have correlated IFN response with substitutions in a region of the nonstructural 5A (NS5A) gene product of HCV, referred to as the interferon sensitivity-determining region (ISDR; position 2209–2248) (Enomoto *et al.,* 1995, 1996; Gerotto *et al.,* 2000; Halfon *et al.,* 2000; Watanabe *et al.,* 2001; Witherell and Beineke, 2001). Such a strict correlation between ISDR mutations and treatment efficacy has not, however, been verified in other studies (Hofgartner *et al.,* 1997; Khorsi *et al.,* 1997; Pawlotsky *et al.,* 1998; Rispeter *et al.,* 1998; Squadrito *et al.,* 1997, 1999; Zeuzem *et al.,* 1997; Sarrazin *et al.,* 1999; Nousbaum *et al.,* 2000). Expression of genotype-1b NS5A protein is sufficient to partially inhibit the antiviral activity of IFN-α in several cell types (Aizaki *et al.,* 2000; Paterson *et al.,* 1999; Polyak *et al.,* 1999; Song *et al.,* 1999), including hepatoma cells (Podevin *et al.,* 2001). Surprisingly, this inhibitory effect of NS5A did not correlate with clinical response (Aizaki *et al.,* 2000; Paterson *et al.,* 1999; Podevin *et al.,* 2001). It has been reported that NS5A binds the dimerization domain of the IFN-inducible dsRNA-dependent protein kinase (PKR) and abolishes its catalytic activity (Gale *et al.,* 1997, 1998). However, we and others suggested that PKR may not mediate the inhibition by NS5A of the IFN antiviral activity (Francois *et al.,* 2000; Podevin *et al.,* 2001). Therefore, despite numerous studies, the mechanisms of viral resistance to IFN-α therapy remain largely undefined. Here we used microarray technologies to identify gene expression profiles of IFN-treated hepatocytic cells expressing NS5A natural mutants isolated from patients resistant or responsive to IFN therapy.

RESULTS

Gene expression profile in IFN-treated Huh7 cells

We wished to compare the interferon responsiveness in Huh7 cells expressing a NS5A mutant isolated from a

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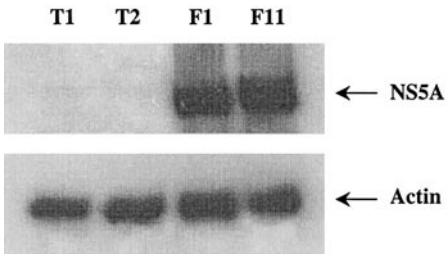

FIG. 1. NS5A expression in Huh7 cell clones. Total proteins from Huh7 clones stably expressing NS5A (F1 and F11) and from control clones (T1 and T2) were separated by SDS–PAGE, transferred to nitrocellulose membrane, and probed with anti-NS5A and anti-β-actin antibodies.

patient infected with HCV genotype 1b, who was a nonresponder to IFN therapy. We previously established stable clones expressing this NS5A sequence and reported that the antiviral activity of IFN against vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) is inhibited in these cells compared to control clones expressing the empty vector (Podevin *et al.,* 2001). In the present study, we used two control clones transfected with the empty vectors (T1 and T2) and two clones stably expressing NS5A (F1 and F11) (Podevin *et al.,* 2001). The expression level of NS5A in these cells is shown in Fig. 1. In two independent experiments, T1 and T2 Huh7 control clones were treated with IFN-α for 24 h. RNA transcript levels for different genes were determined in untreated and IFN-treated cells, using oligonucleotide arrays. Transcripts for ~40% of the 6300 unique genes assessed were detected in Huh7 cells. We identified a subset of genes that significantly differed in their expression levels following IFN treatment by twofold or greater, in both control clones and in all experiments (P value <0.05). The 53 genes identified are presented in Table 1. The large majority of the modified genes (50 of 53) were up-regulated upon IFN-α treatment and represented genes known to be inducible by IFN. This group included the following: Mx1, 2′,5′-oligoadenylate synthetase 1 and 2 (OAS-p40/46, OAS-p69), the protein kinase PKR, signal transducer and activator of transcription 1 (STAT1), interferon-stimulated transcription factor 3 (ISGF3), interferon-induced proteins 15, 20, 27, 35, 41, 56, and 75, promyelocytic leukemia 1 and 2 (PML1, PML2), MHC class I molecules, Mac-2 binding protein (Mac-2bp), phospholipid scramblase, N-myc (and STAT) interactor (NMI), and beta-2 microglobulin. Some of the IFN-regulated genes found were not previously known to be modified by interferon and included the small inducible (Cys-Cys) cytokine LARC (also designated Exodus 1 or MIP-3a) and the TNF-inducible protein A20.

Effect of NS5A on IFN-regulated genes

A similar analysis was performed on the NS5A-expressing Huh7 clones F1 and F11. Genes showing significant differences in response to IFN-α between NS5A-expressing cells and controls (P value <0.05) were selected. Expression of most of the IFN-regulated genes was modified similarly by IFN-α, in NS5A-expressing cells and in control cells. These included PKR, Mx1, and OAS-p40/46, in agreement with our preliminary observation (Podevin *et al.,* 2001). Of the 53 genes, 9 failed to be induced by IFN to the same extent in NS5A-expressing clones compared to controls (Table 2). Induction of Mac-2bp, HLA-B, phospholipid scramblase, LARC, NMI, ISG20, and complement component 3 by IFN was reduced by approximately two- to threefold, whereas no induction of OAS-p69 and A20 was observed in NS5A-expressing cells following IFN treatment.

To validate the microarray data, semiquantitative or real-time PCR amplifications were undertaken for Mac-2bp, phospholipid scramblase, OAS-p69, OAS-p40/46, ISG20, and LARC transcripts (Fig. 2). The PCR and microarray data were highly concordant for these six genes. Mac-2bp is not expressed in untreated Huh7 cells and its induction by interferon is reduced by 2-fold in NS5A-expressing cells compared to control cells. Phospholipid scramblase is expressed in untreated cells and its induction is also reduced by 2-fold in NS5A-expressing cells compared to control cells. We compared IFN induction of OAS-p40/46 and OAS-p69. By microarray, we observed an up-regulation of OAS-p40/46 expression by 22-fold in IFN-treated, control, and NS5A-expressing cells. Similar data were obtained by RT-PCR. In contrast, OAS-p69 expression is induced by IFN-α in control cells but not in NS5A-expressing cells as demonstrated by microarray and RT-PCR experiments. A reduced induction of ISG20 and LARC transcripts in NS5A-expressing cells was also demonstrated by RT-PCR and real-time PCR, respectively. However, this reduction was largely due to an increased expression of both ISG20 and LARC genes in NS5A-expressing cells compared to control cells, in the absence of any IFN treatment.

Effect of NS5A on gene expression profile in Huh7 cells

Our previous data suggested that NS5A also modifies the expression of some IFN-regulated genes in the absence of IFN treatment. Thus, we analyzed the effect of NS5A expression in Huh7 cells, using oligonucleotide arrays. To examine the role of NS5A of gene expression in Huh7 cells, we compared transcript levels in the two F1 and F11 NS5A-expressing clones and in the two T1 and T2 control clones, using oligonucleotide microarrays. Two independent experiments were performed and selection was based on a P value of 0.05 or less. Approximately 100 genes were significantly up- or down-regulated in the NS5A-expressing Huh7 clones. These genes included IFN-inducible genes or genes known to modulate IFN activity, such as ISG20 and LARC, which

TABLE 1

Regulated mRNAs in IFN-Treated Huh7 Cells

GenBank Accession No.	Description	Fold change
M33882	Interferon-inducible protein p78 (Mx1)	55
M13755	Interferon-stimulated protein, 15 kDa (ISG15)	43
J04164	Interferon-induced transmembrane protein 1 (9–27)	~39.5
M24594	Interferon-induced protein 56	~29
L13210	Mac-2 binding protein (Mac-2bp)	~25
X67325	Interferon-α-inducible protein 27	~24
X02874	2′,5′-Oligoadenylate synthetase 1 (OAS-p40/46)	~22
U22970	Interferon α-inducible protein (G1P3)	~16
X57351	Interferon-induced transmembrane protein 2 (1-8D)	~13
U72882	Interferon-induced protein 35	~13
AF008445	Phospholipid scramblase	12.5
D28137	Bone marrow stromal cell antigen 2 (BST2)	~12
X58536	Major histocompatibility complex, class I, C (HLA-C)	9.5
D49824	Major histocompatibility complex, class I, B (HLA-B)	~9
M97936	Signal transducer and activator of transcription 1, 91 kDa (STAT1)	8
M87503	Interferon-stimulated transcription factor 3, γ (48 kDa) (ISGF3)	7
U50648	Interferon-inducible RNA-dependent protein kinase (PKR)	5
M87434	2′-5′-Oligoadenylate synthetase 2 (OAS-p69)	5
U52513	RIG-G	~4.5
M55542	Guanylate binding protein 1, interferon-inducible, 67 kDa (GBP1)	4.5
X04729	Plasminogen activator inhibitor, type 1	3.5
M74447	ATP-binding cassette, subfamily B, member 3	~3.4
X02530	IP10	~3
L22343	Interferon-induced protein 75, 52 kDa	~3
X62741	Proteasome subunit, β type, 9	~3
L22342	Interferon-induced protein 41, 30 kDa	2.8
U34605	Retinoic acid- and interferon-inducible protein, 58 kDa (RI58)	2.6
M83667	CCAAT/enhancer binding protein (C/EBP), δ	2.5
V00594	Metallothionein 2A	2.5
M20022	Major histocompatibility complex, class I, E (HLA-E)	2.5
D50919	KIAA0129	2.5
U32849	N-myc (and STAT) interactor (NMI)	2.4
D32129	Major histocompatibility complex, class I, A (HLA-A)	2.4
D45248	Proteasome activator subunit 2 (PA28 β)	2.3
U88964	Interferon stimulated gene, 20 kDa (ISG20)	2.3
M79463	Promyelocytic leukemia PML-2	2.3
M37435	Colony-stimulating factor 1 (CSF1)	2.2
U64197	LARC/MIP-3a/Exodus 1	2.2
M24283	Intercellular adhesion molecule 1 (ICAM1)	2.2
M79462	Promyelocytic leukemia PML-1	2.2
X90846	Mitogen-activated protein kinase kinase kinase 10 (MAP3K10)	~2.2
L07633	Proteasome activator subunit 1 (PA28 α)	2.1
U37518	TNF-related apoptosis-inducing ligand (TRAIL)	~2.1
M59465	TNF α-induced protein 3 (A20)	2.1
K02765	Complement component 3	2
J04080	Complement component 1	2
S82297	β-2 microglobulin	2
U10439	Adenosine deaminase (ADAR)	2
Z29083	5T4 oncofetal trophoblast glycoprotein	2
Z36714	Cyclin F	~−3.5
X15882	Collagen, type VI, α 2	~−3
M11437	Kininogen	−2.1

Note. RNA from untreated and IFN-treated T1 and T2 Huh7 clones were hybridized onto Affymetrix oligonucleotide arrays and quantified as indicated under Materials and Methods. For each gene, the fold change was calculated by Affymetrix software. The ~ indicates fold change calculation for which the smaller value is replaced by an estimate of the minimum value for detectable transcripts.

showed a 2-fold increase. Additionally, we observed a 5-fold induction of interleukin-8 (IL-8) expression and a 2-fold down-regulation of STAT6 in NS5A-expressing cells. Real-time PCR experiments were performed on the samples analyzed by microarrays. These results confirmed the regulation of IL-8 and STAT6 by NS5A (Fig. 3).

TABLE 2

mRNAs Differently Regulated by IFN in NS5A-Expressing Huh7 Cells

GenBank Accession No.	Description	Fold change T1/T2	Fold change F1/F11	*P* value
L13210	Mac-2 binding protein (Mac-2bp)	~25	~13.5	0.027
D49824	Major histocompatibility complex, class I, B (HLA-B)	~15.5	~6	0.002
AF008445	Phospholipid scramblase	12.5	6.5	0.005
M87434	2′-5′-Oligoadenylate synthetase 2 (OAS-p69)	5	1.2	0.002
U32849	N-myc (and STAT) interactor (NMI)	2.4	1.8	0.035
U88964	Interferon-stimulated gene (20 kDa) (ISG20)	2.3	1.2	0.037
U64197	LARC/Exodus 1/Mip-3a	2.2	1.3	0.012
M59465	Tumor necrosis factor, α-inductible protein 3 (A20)	2.1	1.1	0.014
K02765	Complement component 3	2	1.4	0.025

Note. RNA from untreated and IFN-treated F1 and F11 Huh7 clones was hybridized onto Affymetrix oligonucleotide arrays and quantified as indicated under Materials and Methods. For each gene, the fold change was calculated by Affymetrix software. Genes differently modulated by IFN in these clones compared to T1 and T2 clones ($P < 0.05$) were selected. The ~ indicates the fold change calculation for which the smaller value is replaced by an estimate of the minimum value for detectable transcripts.

Following normalization with β-actin mRNA levels, we observed up-regulation of IL-8 mRNA expression by 9- and 3.4-fold in F1 and F11 clones, respectively. STAT6 expression was decreased by 1.7- and 3.2-fold in F1 and F11 clones, respectively.

Transient transfection assays were performed as a

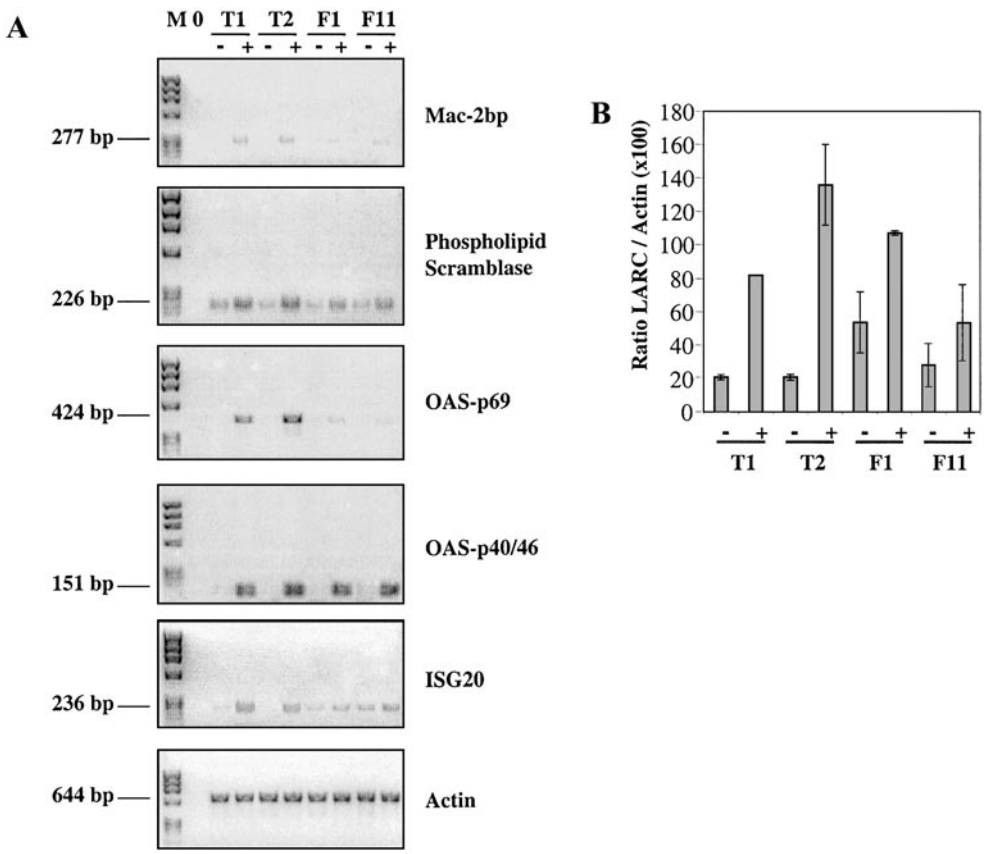

FIG. 2. Effect of NS5A on the expression of some IFN-inducible genes. (A) Total RNA from T1, T2, F1, and F11 Huh7 clones, treated for 24 h with medium alone (−) or with IFN-α (+), was reverse-transcribed and PCR amplification was performed for Mac-2bp, phospholipid scramblase, OAS-p69, OAS-p40/46, ISG20, and actin genes as described under Materials and Methods. Lane (0) represents the negative control, which was the product of PCR where RNA was omitted. (B) LARC mRNA levels were measured in the same cells by real-time quantitative RT-PCR, using the Roche system. The data are presented as ratios of LARC mRNA to β-actin mRNA.

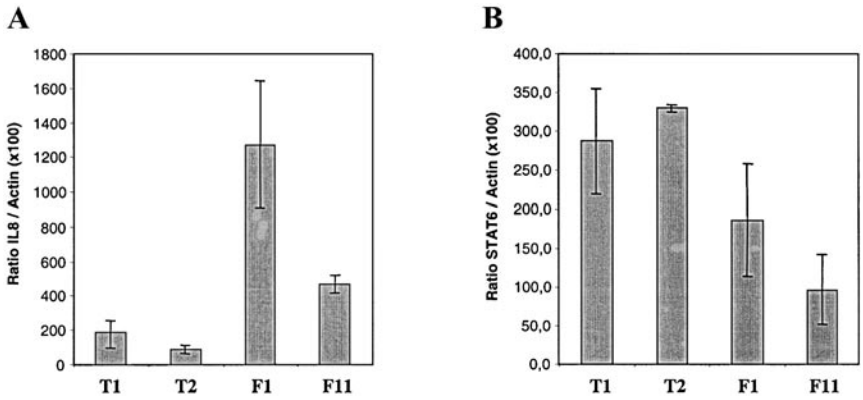

FIG. 3. Effect of NS5A on the expression of IL-8 and STAT6 mRNA. IL-8 and STAT6 mRNA levels were measured in T1, T2, F1, and F11 clones by real-time quantitative RT-PCR, using the Roche system. The data are presented as ratios of IL-8 mRNA (A) or STAT6 mRNA (B) to β-actin mRNA.

direct approach to determine the effect of NS5A on the expression of IL-8, STAT6, LARC, and ISG20. Three NS5A expression plasmids, including NR1 expressed in F1 and F11 cells and NR2 and R1 corresponding to full-length NS5A sequences isolated from a nonresponder and a responder patient, respectively (Podevin *et al.,* 2001), were transfected into Huh7 cells. Transfection with a construct encoding the green fluorescence protein demonstrated a transfection efficiency of approximately 50–60%. NS5A protein expression was analyzed 48 h post-transfection, by Western blotting (Fig. 4). RNA levels were analyzed by microarray analysis, in two independent experiments. Expression of STAT6, LARC, and ISG20 did not change in Huh7 cells upon transient expression of any NS5A sequence, NR1, NR2, or R1. However, IL-8 expression was significantly induced ($P = 0.009$) in Huh7 cells transiently expressing NS5A NR1 compared to control cells expressing the vector alone (data not shown). A similar induction was also observed in cells expressing the NS5A mutants NR2 and R1.

Expression of IL-8 protein in Huh7 cells expressing NS5A

We performed further analysis of the expression of IL-8 in the NS5A-expressing clones F1 and F11. The level of IL-8 protein was quantified by ELISA after 24, 48, and 72 h of culture (Fig. 5). The IL-8 level did not differ between the two control clones T1 and T2, slightly rising with increased time in culture. The IL-8 pattern of expression in these cells was characterized by a slight increase during the culture. In contrast, we observed a strong increase in the IL-8 level at 48 and 72 h in the supernatant of the two clones, F1 and F11, expressing NS5A. After 72 h, IL-8 accumulated in the supernatant of control cells, reaching 4200 and 2800 pg/ml in T1 and T2, respectively. Concentrations of IL-8 were 9600 and 16,000 pg/ml in F1 and F11 clones, respectively.

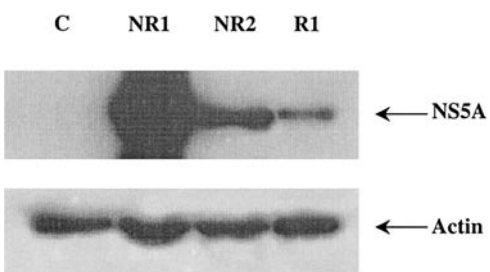

FIG. 4. NS5A expression in Huh7 cells following transient transfections. Huh7 cells were transfected with NR1, NR2, and R1 NS5A-expressing plasmids or with the empty vector (C). After 48 h, total proteins were separated by SDS–PAGE, transferred to nitrocellulose membrane, and probed with anti-NS5A and anti-β-actin antibodies.

Effect of IL-8 on IFN-mediated antiviral activity in Huh7 cells

It has been reported that IL-8 reduces the antiviral activity of IFN-α in fibroblastic cells (Khabar *et al.,* 1997). To examine the potential effect of IL-8 on the IFN-mediated antiviral activity in hepatoma cells, viral rescue assays were performed using VSV as the challenge

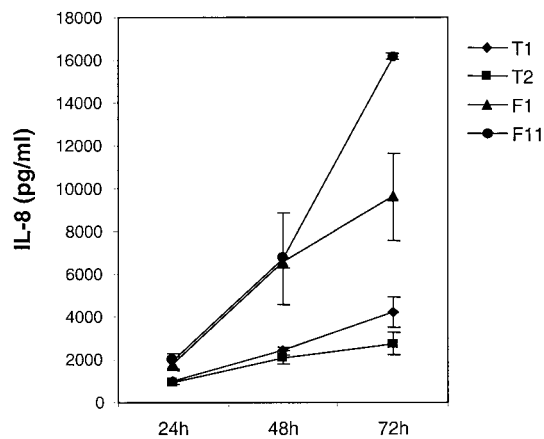

FIG. 5. Expression of IL-8 protein in NS5A-expressing cells as determined by ELISA. T1, T2, F1, and F11 Huh7 clones were cultured for 3 days and IL-8 was measured in the supernatants every 24 h, by ELISA.

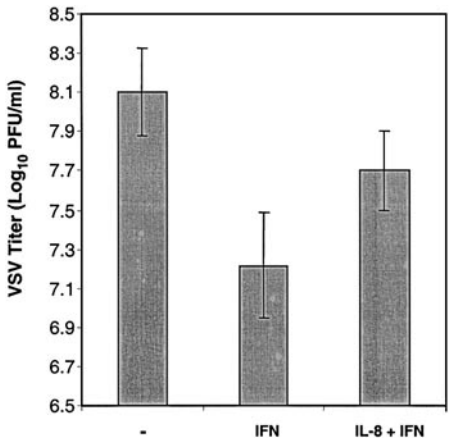

FIG. 6. Effect of IL-8 on VSV challenge in IFN-treated Huh7 cells. Huh7 cells were pretreated with IL-8 followed by IFN-α for 24 h before infection with VSV at a multiplicity of infection of 1 PFU/cell. At 1 day postinfection, virus titers were measured. Bars represent the mean + SEM of four independent experiments.

virus. Huh7 cells were pretreated or not with IL-8 for 20 h prior to addition of IFN-α. Cells were then infected with VSV for 24 h and virus titers were measured by $TCID_{50}$ assay. We found that IFN-α inhibited replication of VSV by 0.9 log. In contrast, the antiviral activity of IFN was reduced by 3.2-fold in the presence of IL-8 (Fig. 6). Thus, IL-8 partially inhibits the antiviral activity of IFN-α in hepatocytic cells.

Effect of IL-8 on specific IFN-regulated genes

We wished to investigate the role of IL-8 up-regulation by NS5A, in the reduced induction by IFN-α observed on selective genes in NS5A-expressing Huh7 cells. Semi-quantitative PCR amplifications were undertaken for Mac-2bp, phospholipid scramblase, OAS-p69, ISG20, and LARC transcripts. Induction of Mac-2bp, phospholipid scramblase, OAS-p69, and ISG20 following IFN-α treatment was reduced in IL-8-pretreated Huh7 cells compared to control cells (Fig. 7). In addition, IL-8 induced ISG20 expression, similarly to NS5A. No effect of IL-8 on the induction of LARC expression by IFN-α was observed (data not shown). We conclude that up-regulation of IL-8 by NS5A partially mediates the reduction of IFN induction observed in NS5A-expressing cells, for selected IFN-inducible genes.

DISCUSSION

Several studies demonstrated a major role of the nonstructural HCV NS5A protein in IFN resistance (Enomoto *et al.,* 1996; Gale *et al.,* 1997; Paterson *et al.,* 1999; Podevin *et al.,* 2001; Polyak *et al.,* 1999; Song *et al.,* 1999). In order to develop a better understanding of the mechanism involved, we utilized oligonucleotide microarrays for a systematic quantitative analysis of IFN-inducible genes in hepatoma cells expressing NS5A. We previously reported that IFN antiviral activity is partially inhibited in these NS5A-expressing Huh7 cells (Podevin *et al.,* 2001). Here, we identified 53 genes regulated by IFN-α treatment. A large number of these genes have been previously identified as being regulated by IFN-α in a fibrosarcoma cell line, using oligonucleotide arrays (Der *et al.,* 1998). Moreover, many genes consisted of known interferon-stimulated genes, as indicated in previously published studies. The remaining genes representing novel IFN-regulated genes include the small inducible cytokine LARC (Exodus-1 or MIP-3a) and the TNF-inducible protein A20.

Nine of the 53 genes identified in control cells did not respond to the same extent to IFN-α in cells expressing NS5A ($P < 0.05$). The induction by IFN-α was reduced in NS5A-expressing cells compared to control cells by approximately twofold, for Mac-2bp, HLA-B, phospholipid scramblase, NMI, A20, and complement component

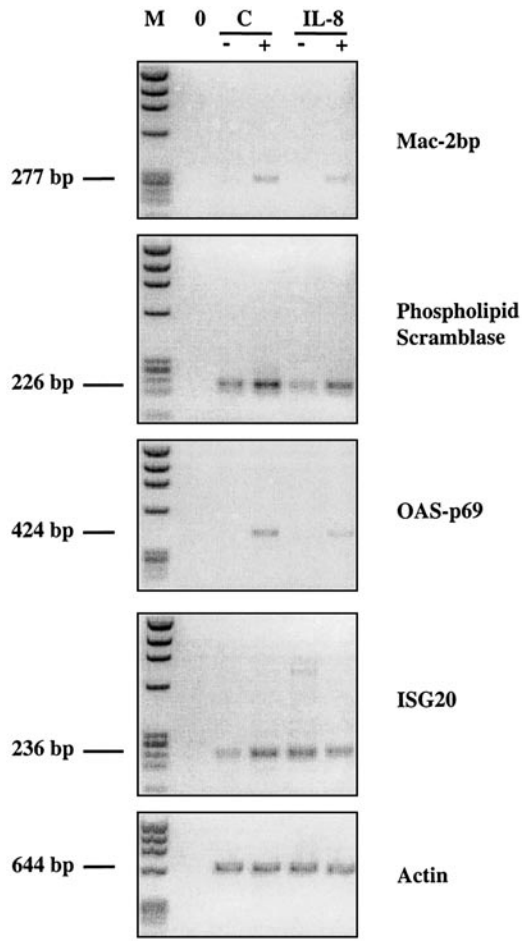

FIG. 7. Effect of IL-8 on the expression of specific IFN-inducible genes. Huh7 cells, control (C) or pretreated with IL-8 (IL-8), were subsequently stimulated for 24 h with medium alone (−) or with IFN-α (+). Total RNA was isolated and reverse-transcribed, and PCR amplification was performed for Mac-2bp, phospholipid scramblase, OAS-p69, ISG20, and actin genes as described under Materials and Methods. Lane (0) represents the negative control, which was the product of PCR where RNA was omitted.

3. No antiviral activity for these genes has been described so far. Elevated levels of Mac-2bp have been found in the sera of patients with various types of cancer and viral infection, as well as in patients with hepatocellular carcinoma and cirrhosis (Correale *et al.,* 1999). Serum Mac-2bp levels are also increased in chronic viral hepatitis patients, being significantly higher in HCV than in HBV patients (Artini *et al.,* 1996). Interestingly, it has been suggested that Mac-2bp levels could be an independent predictor of disease severity and of nonresponsiveness to IFN-α treatment in chronic HCV patients (Artini *et al.,* 1996; Kittl *et al.,* 2000). Hepatocellular expression of HLA-A, B, and C molecules was also reported to predict primary response to interferon in patients with chronic hepatitis C (Ballardini *et al.,* 1994, 1995). Phospholipid scramblase regulates lipoprotein levels and it has been suggested that this protein mediates the redistribution of plasma membrane phospholipids in apoptotic cells (Zhao *et al.,* 1998). NMI, a protein with 25% amino acid identity with the interferon-inducible protein IFP35, interacts with c-Myc, N-Myc, Max, and Fos. NMI and IFP-35 also associate into a high-molecular-mass complex (Chen *et al.,* 2000). The zinc finger protein A20 is an inhibitor of NF-κB activity and TNF-mediated apoptosis (reviewed by Beyaert *et al.,* 2000). Its expression is inducible by a variety of stimuli including cytokines such as TNF and viral products. However, induction of A20 by IFN has not been reported so far. Using A20-deficient mice, a recent study demonstrated that A20 is critical for limiting inflammation by terminating TNF-induced NF-κB responses *in vivo* (Lee *et al.,* 2000). The liver is the major expression site of complement component 3, the central mediator of complement activation. The number of viruses found to interact with molecules of the complement system is growing and the widespread presence of complement-modifying proteins in pathogenic viruses suggests that they may play an important role in disease progression. The inhibition of C3 expression by NS5A in IFN-treated cells suggests a possible immunoregulatory role of HCV NS5A protein.

The 2′,5′-oligoadenylate synthetases (OAS) represent a family of IFN-induced proteins implicated in the antiviral action of IFN. Three forms of human OAS have been described and are encoded by three distinct genes (Hovanessian *et al.,* 1987; Hovnanian *et al.,* 1998). The different forms of OAS have distinct enzymatic parameters, suggesting that they might have different specific functions (Marie *et al.,* 1997; Rebouillat and Hovanessian, 1999). In contrast to OAS-p40/46 mRNA, OAS-p69 mRNA failed to be induced by IFN in cells expressing NS5A. Interestingly, it has been reported that OAS-p69 can mediate the antiviral action of IFN (Marie *et al.,* 1999). The promoter region of the genes encoding OAS-p40/46 and OAS-p69 have a typical functional IFN-stimulated response element but the OAS-p69 promoter is more complex and at least three regulatory elements cooperate to give a maximal activation after treatment by IFN (Floyd-Smith *et al.,* 1999; Wang and Floyd-Smith, 1998). The precise role of OAS-p69 in IFN resistance induced by NS5A remains to be determined.

In the absence of any IFN treatment, NS5A also modified the expression of genes modulated by IFN or known to modulate IFN activity. These included ISG20, LARC, STAT6, and IL-8. ISG20 is closely associated with the PML and SP100 genes (Gongora *et al.,* 1997). LARC (also called Exodus 1 or MIP-3a) is a chemokine that was recently characterized, expressed mainly in liver. LARC is up-regulated by mediators of inflammation such as TNF and is significantly chemotatic for lymphocytes (Hieshima *et al.,* 1997). STAT6 mediates responses to IL-12 and IL-4 (Takeda and Akira, 2000) and it has been shown that IFN inhibits the IL-4 and IL-13 induction of a few genes, by inhibiting STAT6 activity (Dickensheets and Donnelly, 1999). In addition, STAT6 mediates suppression of STAT1 and NF-κB-dependent transcription (Ohmori and Hamilton, 2000). Most interestingly, we observed a strong up-regulation of IL-8 mRNA and protein expression in Huh7 cells expressing NS5A. This is in agreement with recent data demonstrating an induction of IL-8 expression by NS5A in HeLa cells (Polyak *et al.,* 2001a). It was also reported that IL-8 reduces the antiviral activity of IFN-α in fibroblasts and HeLa cells (Khabar *et al.,* 1997). We demonstrated a similar inhibition of IFN antiviral activity by IL-8 in hepatocytic Huh7 cells. IL-8 expression is induced by several viruses including human herpesvirus 6 (Inagi *et al.,* 1996), cytomegalovirus (Murayama *et al.,* 1998), and respiratory syncitial virus (Becker *et al.,* 1991; Black *et al.,* 1998; Fiedler *et al.,* 1995, 1996) through NF-κB and AP-1 signaling (Casola *et al.,* 2000; Mastronarde *et al.,* 1996, 1998). It has been reported that IL-8 is significantly elevated in patients with chronic HCV infection and that the level of IL-8 correlates with liver fibrosis (Kaplanski *et al.,* 1997). IL-8 levels are also more elevated in sera of patients with hepatocellular carcinoma (HCC) and chronic active hepatitis associated with HCV (Al-Wabel *et al.,* 1995; Miyamoto *et al.,* 1998). Additionally, cytotoxic lymphocytes from patients infected with HCV produce IL-8 (Koziel *et al.,* 1995). Remarkably, elevated levels of IL-8 in HCV-infected patients, with significantly higher IL-8 levels in patients who did not respond to IFN therapy, were recently reported (Polyak *et al.,* 2001b). HCV core protein activates the IL-8 promoter through NF-κB and AP-1 (Kato *et al.,* 2000). Here we show that the induction of IL-8 may also be dependent on NS5A in hepatocytic cells. Induction of IL-8 mRNA by NS5A was observed 48 h posttransfection and remarkably, all three NS5A sequences tested, obtained from patients either responsive or not to IFN therapy, did induce IL-8 expression.

The release of IL-8 induced by HCV viral proteins such as NS5A may contribute to the pathogenesis of HCV-induced liver damage as well as to the mechanisms of

IFN resistance. Remarkably, all three NS5A sequences analyzed, which were derived from patients who were responders and nonresponders to IFN therapy, induced IL-8 expression, suggesting that other mechanisms are important for the final phenotypic effect of NS5A. This emphasizes the relevance of a global approach to study NS5A-related biological effects. We also demonstrated that NS5A inhibited the induction by IFN of selected genes including OAS-p69. These effects may also contribute to the resistance to IFN mediated by NS5A. In addition, NS5A modifies various activities in response to IFN that would be predicted to provide survival value for the virus by subverting host defense and apoptotic mechanisms.

MATERIALS AND METHODS

Cell culture

The human hepatoma Huh7 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. We recently established stable Huh7 clones expressing three NS5A mutants (Podevin *et al.,* 2001). These three NS5A sequences were obtained from patients infected with genotype 1b HCV who were responders or nonresponders to IFN therapy. Herein, we used the control clones T1 and T2 and two stable clones, F1 and F11, expressing the NS5A mutant NR1, isolated from a nonresponder patient (Podevin *et al.,* 2001). The established clones were grown in DMEM containing 10% FCS and 400 μg/ml of geneticin (Gibco BRL). When indicated, the cells were treated with recombinant IFN-α (Schering-Plough) for 24 h at a concentration of 100 U/ml or with hIL-8 (R&D Systems) for 24 h at a concentration of 0.15 μg/ml.

Transient NS5A expression

Huh7 cells were plated in 100-mm dishes at 4×10^5 cells/dish. Twenty-four hours later, the cells were transfected by addition of 30 μl of the Lipofectamine transfection reagent (Gibco BRL) and 15 μg of the plasmid containing the NR1, the NR2, or the R1 NS5A sequence or of the empty vector, in OPTIMEM I medium (Gibco BRL). NR1 and NR2 represent NS5A natural mutants isolated from patients infected with HCV-1b and who were nonresponders to IFN therapy, whereas R1 corresponds to an NS5A natural mutant isolated from a patient who responded to IFN therapy (Podevin *et al.,* 2001). The cells were cultured for an additional 48 h in DMEM supplemented with 7.5% FCS. In additional experiments, Huh7 cells were cotransfected with 4 μg of the pEGFP vector encoding the green fluorescence protein (Clontech, France) as a monitor for transfection efficiency.

Preparation of cRNA and gene chip hybridization

Total RNA was isolated using Trizol reagent (Gibco BRL), followed by clean-up on an RNeasy spin column (Qiagen), and then used to generate cRNA probes. Preparation of cRNA, hybridization, and scanning of the HuGeneFL arrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Briefly, 5 μg of total RNA was converted into double-stranded cDNA by reverse transcription using a cDNA synthesis kit (Superscript Choice System, Gibco BRL) with an oligo(dT)24 primer containing a T7 RNA polymerase promoter site added 3′ of the poly(T) (Genset, La Jolla, CA). Following second-strand synthesis, labeled cRNA was generated from the cDNA sample by an *in vitro* transcription reaction supplemented with biotin-11–CTP and biotin-16–UTP (Enzo, Farmingdale, NY). The labeled cRNA was purified by using RNeasy spin columns (Qiagen). Fifteen micrograms of each cRNA was fragmented at 94°C for 35 min in fragmentation buffer (40 mM Tris–acetate (pH 8.1), 100 mM potassium acetate, 30 mM magnesium acetate) and then used to prepare 300 μl of hybridization cocktail (100 mM MES, 1 M NaCl, 20 mM EDTA, 0.01% Tween 20) containing 0.1 mg/ml of herring sperm DNA (Promega, Madison, WI), 500 μg/ml acetylated BSA (Gibco BRL), and a mixture of control cRNAs for comparison of hybridization efficiency between arrays and for relative quantitation of measured transcript levels. Prior to hybridization the cocktails were heated to 94°C for 5 min, equilibrated at 45°C for 5 min, and then clarified by centrifugation (16,000 *g*) at room temperature for 5 min. Aliquots of each sample (10 μg of fragmented cRNA in 200 μl of hybridization cocktail) were hybridized to HuGeneFL arrays at 45°C for 16 h in an oven set at 60 rpm. The arrays were then washed with nonstringent wash buffer (6X SSPE) at 25°C, followed by a wash with stringent wash buffer (100 mM MES (pH 6.7), 0.1 M NaCl, 0.01% Tween 20) at 50°C, stained with streptavidin–phycoerythrin (Molecular Probes), washed again with 6X SSPE, and stained with biotinylated anti-streptavidin IgG, followed by a second staining with streptavidin–phycoerythrin and a third washing with 6X SSPE. The arrays were scanned using the GeneArray scanner (Affymetrix). Data analysis was performed using GeneChip 4.0 software. The software includes algorithms that determine whether a gene is absent or present and whether the expression level of a gene in an experimental sample is significantly increased or decreased relative to a control sample.

SDS–PAGE and Western blotting

Cells were lysed in 20 mM Tris–HCl, pH 7.5, buffer containing 5 mM EDTA and 100 mM KCl. Total proteins (50 μg) were diluted in Laemmli sample buffer, resolved by SDS–PAGE in a 9% polyacrylamide gel, and transferred onto a 0.22-μm nitrocellulose membrane (Schlei-

TABLE 3

Primer Sequences

Gene	Sequence	Product size (bp)	Annealing temperature (°C)
Actin	5′-ATCATGTTTGAGACCTTCAA-3′ 5′-TTG CGCTCAGGAGGAGCAAT-3′	644	60
IL-8	5′-ATGACTTCCAAGCTGGCCG-3′ 5′-CTCAGCCCTCTTCAAAAACTT-3′	291	62
Mac-2bp	5′-GCGAGGAGGCTCCACACGG-3′ 5′-GGTGGCGTTCTCGAAGCCC-3′	277	60
Phospholipid scramblase	5′-CTTGTCTCGCTCGGGAGCGG-3′ 5′-CTGGGCCAGGACCTGAATGG-3′	226	60
OAS-p69	5′-TTAAATGATAATCCCAGCCC-3′ 5′-AAGATTACTGGCCTCGCTGA-3′	424	60
OAS-p40/46	5′-AGTTCTGTTGCCACTCTCTCTC-3′ 5′-TCAGGAACCCACAGATGATG-3′	151	62
ISG20	5′-GGTGGTGGCCATGGACTGC-3′ 5′-GCTTGCCTTTCAGGAGCTGC-3′	236	60
LARC	5′-CAAGAGTTTGCTCCTGGCTGC-3′ 5′-CAGCTGCCGTGTGAAGCCC-3′	148	60
STAT6	5′-CAGAGCTACAGACCTATGGGG-3′ 5′-GAAGATGCCGCAGGTGTTGG-3′	180	60

cher and Schuell, Dasel, Germany). The membranes were incubated for 2 h in blocking buffer containing 5% milk in 10 mM Tris–HCl, pH 7.5; 2.5 mM EDTA, pH 8; 50 mM NaCl. Subsequent incubation was performed for 2 h with monoclonal antibodies against NS5A (Biogenesis) (1/1000) or actin (ICN) (1/20,000). After three washes, the membranes were finally incubated for 1 h with a horseradish peroxidase-conjugated anti-mouse antibody (Amersham) (1/2000). Immunodetection was realized by enhanced chemiluminescence reagents (Amersham) followed by autoradiography on hyperfilm MP (Amersham).

RT-PCR

Single-stranded cDNA was synthesized from total RNA using Superscript II reverse transcriptase (Gibco) and random hexamers (Gibco) as primers. Preliminary experiments were performed to determine the conditions in which cDNAs were amplified in the linear region of the PCR curve. The reaction mixture was composed of 1 μl of cDNA template (diluted 1/5) obtained from 1 μg of extracted RNA; 25 pmol of primers; 25 nmol of each dNTP; 2.5 U of *Taq* DNA polymerase (Gibco); 10 μl of 10× PCR buffer in a final volume of 50 μl. The nucleotide sequence of primers and corresponding annealing temperatures used are as indicated (Table 3). PCR amplification conditions were as follows: denaturation at 94°C for 5 min, amplification for 25, 30, or 35 cycles composed of denaturation at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The expression level for each transcript was evaluated following ethidium bromide staining by densitometric scanning and analysis using Image Quant Software.

For real time RT-PCR, total RNA was amplified by RT-PCR using a LightCycler instrument (Roche Molecular Biochemicals), which amplifies and monitors by fluorescence assay the development of target nucleic acid after each cycle. The RT-PCRs were performed in capillaries (Roche) with 200 ng of total RNA in a total volume of 20 μl using the LightCycler-RNA amplification Kit SYBR green I (Roche) and according to the manufacturer's instructions. The amplification steps were as follows: reverse transcription for 10 min at 55°C, denaturation for 1 min at 95°C, 45 cycles consisting of denaturation for 1 s at 95°C, annealing for 8 s at 60°C, and extension for 15 s at 72°C. Analysis of the PCR amplification and probe melting curves was accomplished through the use of LightCycler software. Actin mRNA was used to normalize the amounts of input mRNAs in each sample.

Quantitative enzyme-linked immunosorbent assay (ELISA) for interleukin-8

Amounts of IL-8 present in the culture medium of the T1, T2, F1, and F11 Huh7 clones were measured by ELISA with the Quantikine human IL-8 kit (R&D Systems), after 24, 48, and 72 h of culture. Aliquots of 50 μl of diluted (1/10 and 1/100) and undiluted media were used for ELISA according to the manufacturer's instructions. The captured IL-8 was then quantitated at 450 nm in a spectrophotometer. Two independent experiments as well as two independent measures were performed for each sample.

Virus rescue assay

Stocks of VSV were prepared from the supernatants of virus-infected L929 mouse fibroblast cells and exhibited

titers of 1.05×10^7 plaque-forming units (PFU) per milliliter. Huh7 cells were seeded in 6-well tissue culture plates at a density of 2×10^5 cells/well. Recombinant human IL-8 (R&D Systems) was added to the cells for 20 h at a concentration of 0.15 μg/ml, followed by recombinant IFN-α (Schering-Plough) for 24 h at a concentration of 200 U/ml. Cells were then infected with 0.1 ml of VSV with a multiplicity of infection of 1 PFU/cell. After adsorption for 30 min at room temperature, excess virus was removed by washing with serum-free medium, and fresh medium containing 2% FCS was added. At 24 h postinfection, the supernatants were collected and clarified. Following serial dilutions at 1/10, virus titers were measured by $TCID_{50}$ assays on L929 cells. Four independent experiments were performed.

ACKNOWLEDGMENTS

This work was supported in part by NIH Grant DK58771, by a grant from the University of Michigan Biomedical Research Council, and by a grant from the University of Michigan Gastrointestinal Peptide Research Center (2 P30 DK34933). The Association de Recherche contre le Cancer (ARC) is acknowledged for the fellowship accorded to SG.

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